Autonomic conflict exacerbates long-QT associated ventricular arrhythmia

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Abstract (250 words)

The present study tested the hypothesis that concomitant sympathetic and parasympathetic stimulation ("autonomic conflict") may act as a trigger for arrhythmia in long QT syndrome (LQTS). Studies were performed in isolated innervated rabbit hearts treated with clofilium (100nmol/L); a potassium channel blocker. The influence of vagus nerve stimulation (VNS) on spontaneous ventricular arrhythmia was assessed in the absence/presence of sustained noradrenaline perfusion (100nmol/L) and with sudden adrenergic stress (injections of noradrenaline into the perfusion line). Hearts were instrumented for a pseudo-electrocardiogram and monophasic action potential recordings. VNS, which slows heart rate, was associated with a stimulation frequency-dependent incidence of spontaneous early afterdepolarisations (EADs) and ventricular tachycardia (VT), best predicted by the duration of the electrocardiographic T-wave and by triangulation of the ventricular action potential. In the presence of sustained (steady-state) noradrenaline perfusion, the incidence of EADs and VT with VNS was decreased from 73/55% to 45/27%, respectively. However, sudden adrenergic stress, imposed during periods of sustained VNS, was associated with a transient increase in the incidence of severity of observed arrhythmia, as indicated by an increase in the average arrhythmia score (1.6±0.4 vs. 2.1±0.7, p=0.01). Analysis of electrophysiological parameters suggests that sudden adrenergic stress is associated with a transient prolongation, and increased triangulation, of the ventricular action potential, which may predispose to triggered activity. This study demonstrates that autonomic conflict is pro-arrhythmic stimulus in LQTS. However, combined adrenergic and parasympathetic stimulation has a complex relationship with arrhythmogenicity, with differences in the effects of steady-state adrenergic activation vs. sudden adrenergic stress.

Highlights

- Long QT syndrome associated arrhythmia were studied with dual autonomic stimulation
- Sudden adrenergic stress increased the severity of arrhythmia
- Sustained adrenergic stimulation suppressed arrhythmia
- Changes in electrical parameters mimic profile the temporal profile of arrhythmia

Keywords

autonomic conflict; long QT syndrome; torsades de pointes; sympathetic; parasympathetic; autonomic nervous system

Abbreviations

EAD, early afterdepolarisation; LQTS, long QT syndrome; TdP; torsades de pointes; TpTe; T-wave peak-to-end interval; VT, ventricular tachycardia; VNS, vagus nerve stimulation.

1. Introduction

Malignant ventricular arrhythmias (e.g. torsades de pointes - TdP) are the primary cause of syncope and premature death in patients with abnormal prolongation of the electrocardiographic QT interval. Despite extensive research, the role of the autonomic nervous system in long QT syndrome (LQTS) associated arrhythmogenesis is imperfectly understood. The conventional textbook picture of the cardiac autonomic control suggests the two branches of the autonomic nervous system exert reciprocal actions on the heart, such as in the baroreceptor reflex. In reality, this represents an over-simplification of a system in which simultaneous and synchronous activation often occurs.[1-3] For example, during cold-water immersion, activation of the parasympathetically mediated mammalian diving reflex and sympathetic / adrenergically driven cold-water shock response occurs simultaneously.[1-3]

Cold-water submersion (head under) is known to be a trigger for supraventricular and ventricular arrhythmia in young, seemingly healthy people, who do not exhibit evidence of arrhythmia at rest.[3-7] These arrhythmic events are not commonly observed in the same individuals during head out cold-water immersions (sympathetic stimulation, no parasympathetic stimulations), or during facial immersion, with breath-hold, which activates the parasympathetic diving reflex and not the cold-shock (sympathetic) response. This suggests that simultaneous autonomic activation, as in whole-body cold-water submersion, might be an arrhythmogenic stimulus, and is supported by other anecdotal observations,[1] but has never been tested experimentally. We recently hypothesised that the sudden and simultaneous sympathetic and parasympathetic activity, termed “autonomic conflict”, may be a cause of arrhythmia associated with cold-water submersion and more generally in situations where autonomic co-activation occurs in conjunction with existing predisposing factors.[3]

Post-mortem analysis using DNA sequencing has revealed that nearly 30% of the victims of seemingly unexplained drowning have cardiac ion channel mutations.[8] Moreover, a strong association between swimming and arrhythmia/sudden cardiac death has been shown in children with heritable LQTS and swimming is recognised as a gene-specific trigger for TdP in patients with congenital LQTS type 1.[9-12] Could this represent something more than a simple increase in adrenergic tone? Similarly, patients with LQTS type 2 generally die during times of sudden stress, such as when awoken by an alarm clock, suggesting that it is sympathetic activation during periods of high parasympathetic tone that triggers TdP.[10, 13] Evidence from 24-hour electrocardiogram recordings in patients with acquired LQTS indicates that while prevailing rates are relatively slow, there is a significant increase in heart rate in the minutes preceding electrical instability and TdP.[14] Therefore, we propose that the sequence and timing of autonomic activation plays a critical

role in the induction of LQTS associated arrhythmia. In this study, we examined the role of autonomic conflict as a trigger for ventricular arrhythmia in conditions of acquired (type 2) LQTS. We hypothesised that concomitant sympathetic and parasympathetic stimulation would act as a trigger for LQTS associated arrhythmogenesis.

2. Methods

2.1 Animal welfare

All procedures were undertaken in accordance with ethical guidelines set out by the UK Animals (Scientific Procedures) Act 1986 and Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Studies conformed the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health under assurance number A5634-01. Studies were approved by local ethics review at King's College London.

2.2 Isolated innervated rabbit heart

All studies utilised the isolated innervated rabbit heart as described by Ng *et al*, with minor modifications *[10]*. Experiments were performed in New Zealand White Rabbits (2.5-4.5kg). Animals were pre-sedated using a mixture of ketamine (Ketaset, 10mg/kg, Fort Dodge, UK), medetomidine hydrochloride (Sedator, 0.2mg/kg, Dechra, UK) and butorphanol (Torbugesic, 0.05mg/kg, Fort Dodge, UK) (i.m.). Anaesthesia was induced and maintained using propofol (5mg as required, Rapinovet, Schering-Plough Animal Health, UK), with concomitant heparin (1000 units, Multiparin, UK). Animals were intubated and ventilated at 60 breaths per minute of room air. The major blood vessels leading to and from the thorax were ligated and cut. Animals were killed by an overdose of sodium pentobarbitone solution (160mg/kg i.v.). The descending aorta was cannulated and the preparation excised from C1-T12 (on ice). Hearts were retrogradely perfused with an oxygenated Krebs-Hensleit buffer, containing (in mM): NaCl 114, KCl 4, CaCl 1.8, $NaHCO_3$ 24, $MgSO_4$ 1, NaH_2PO_4 1.1, glucose 11.0 and sodium pyruvate 1.0. Perfusion rate was set to give an aortic pressure of 70-80mmHg. The right vagus nerve was isolated from the surrounding tissues and placed on silver bipolar stimulating electrodes connected to a constant voltage stimulator (DS2A, Digitimer, UK).

2.3 Experimental protocols

All hearts were instrumented for recording of a pseudo bipolar electrocardiogram (low pass=100Hz, high pass=0.3Hz, sampling=1KHz) and monophasic action potential from the left ventricular free wall (low pass=100Hz, high pass=0.3Hz, sampling=1kHz). Hearts were perfused with clofilium tosylate (100nmol/L in DMSO) for 20-minutes prior to the commencement of the experimental protocols and throughout the study. 100nmol/L clofilium was sufficient to cause substantive QT prolongation without AV-block, which can occur with higher concentrations of class III anti-arrhythmic agents.

2.3.1 Protocol 1 (Sustained noradrenaline)

Cardiac responses to VNS were assessed at 2, 5, 10 and 20Hz stimulation [pulse width=1ms, amplitude=10V, duration=2 minutes per frequency] in the absence and presence of 100nmol/L noradrenaline (+50μM ascorbate). This represents a submaximal concentration in the rabbit heart and produces a marked and sustained chronotropic and electrophysiological action. Noradrenaline was perfused for a minimum of 10-minutes prior to nerve stimulation.

2.3.1 Protocol 2 (Bolus noradrenaline)

Noradrenaline (0.1ml of 1mM stock) was injected directly into the bubble trap of the perfusion apparatus at intrinsic heart rates and following 2-minutes of VNS at 5 and 10Hz. Hearts were allowed to recover for 10-minutes between injections.

2.4 Blinding and randomisation

To reduced inter-operator variability, studies were conducted and analysed by a single experimenter. The sequence of experimental protocols and order of stimulations in Protocols 1 and 2 were randomised prior to the study. The nature of the paired study design prevented blinding of the experimenter to the study protocols.

2.5 Data analysis

Experiments were ranked per the severity of observed arrhythmia, using a predefined arrhythmia score (no arrhythmia=1, early afterdepolarisations (EADs)=2, ventricular tachycardia (VT)=3, sustained VT (>20secs) or ventricular fibrillation=4). Note that this is a simple hierarchical scoring system, in which the absolute score at each level is not a true quantification of arrhythmia severity. Curtis and Walker have previously shown the validity of such hierarchical systems for use in arrhythmia quantification.[15] EADs were assessed first in monophasic action potential recordings and then confirmed in ECG recordings - evidenced by an R-T ectopics in the latter and premature beat occurring before repolarisation of the preceding action potential in MAP recordings. Because TdP can present monomorphic or polymorphic patterns in ECG recordings from the same subject, depending on the relative angle of the recording electrode, data are presented in terms of the number of episodes of VT. In most cases, ECG and monophasic action potential parameters were assessed from 10-beat averages of normally conducted beats. Monophasic action potential duration was assessed at 90% repolarisation ($MAPD_{90}$). Action potential triangulation was calculated as $MAPD_{90}$-$MAPD_{30}$.

2.6 Statistics

Arrhythmia scores were compared by Wilcoxon's matched-groups signed rank-sum tests. For electrophysiological parameters, parametric statistical analyses were applied on the basis that the distribution of the QT interval is Gaussian and on the assumption that other electrogram parameters and related measures (e.g. action potential triangulation) are similarly distributed. Statistical comparisons were made using paired Student's t-tests and repeated measures one or two-way ANOVA with Sidak's post-hoc tests, as appropriate. $p<0.05$ was considered significant. Continuous variables are presented as means (standard deviation (SD)).

3. Results

3.1 VNS is a trigger for EADs and VT in LQTS

The representative trace in Figure 1A demonstrates a frequency-dependent slowing of heart rate with VNS in a clofilium-treated rabbit heart. In this example, spontaneous EADs were observed during high-intensity VNS (20Hz). Data presented on the left of Figure 1B&C summarise the incidence of arrhythmia with VNS in 11 experiments, and show that VNS acted as a stimulation frequency-dependent trigger for spontaneous EADs and VT in LQTS; with no arrhythmia observed in baseline conditions (i.e. in hearts beating at their intrinsic rate). The percentage of hearts exhibiting EADs increased from 0% at baseline to 73% with 20Hz VNS, and the maximum incidence of spontaneous VT was 55%. Neither EADs or spontaneous VT were observed with VNS before clofilium perfusion (data not shown). Arrhythmia were suppressed by the restoration of normal beating rates, either spontaneously or by short-term ventricular pacing (after which hearts returned to sinus rhythm).

3.2 T-wave duration and action potential triangulation predict EADs during VNS

Table 1 presents a comparison of electrophysiological parameters in hearts with and without spontaneous EADs during VNS (5Hz) and shows that the duration of the T-wave (TpTe interval), a measure of ventricular dispersion of repolarisation, and triangulation of the ventricular action potential are the best predictors of VNS-associated triggered activity.

3.3 Sustained noradrenaline perfusion supresses long QT associated arrhythmia

In the presence of sustained (steady-state) noradrenaline the propensity for EADs and VT with VNS was substantially reduced (Figure 1B&C). With VNS at 20Hz, 73% of hearts exhibited EADs in control conditions, compared to only 45% in the presence of noradrenaline. TdP exhibited a similar pattern, with an incidence of 55% in control conditions vs. 27% during noradrenaline perfusion. The average arrhythmia score, a measure of the severity of observed arrhythmia, was reduced by sustained noradrenaline perfusion (Figure 1D&E).

3.4 VNS antagonises the action of catecholamines on heart rate but not on ventricular electrophysiology

The electrophysiological consequences of sustained (steady-state) noradrenaline perfusion are presented in Figure 2. As expected, noradrenaline perfusion resulted in an increase in heart rate and shortening of the QT interval, as well as a reduction in the magnitude of action potential triangulation. In the presence of noradrenaline, VNS slowed heart rate to a greater degree than that observed in control conditions. At baseline, and with low intensity VNS (2Hz), heart rate was elevated by noradrenaline, but this effect was lost with at higher

intensities of stimulation (Figure 2B) - showing that the vagus nerve antagonises the action of noradrenaline on heart rate. By comparison, adrenergic-dependent shortening of the QT interval was not reversed by VNS (Figure 2C). In fact, the magnitude of VNS-dependent QT prolongation was less in the presence of catecholamines (68 (5)ms vs. 127 (14)ms, p=0.003) and so QT interval was substantially shorter at all frequencies of nerve stimulation, despite a similar underlying heart rate during high intensity VNS (see Figure 2C). Similarly, a greater increase in ventricular dispersion of repolarisation (as indicated by prolongation of the TpTe interval) and triangulation of the action potential was observed with VNS in the absence of adrenergic stimulation (Figure 2D&E). The TpTe interval increased by 136 (34)% with VNS alone and 64 (22)% with VNS and noradrenaline perfusion (p=0.004). Action potential triangulation increased by 70±16ms with VNS in control conditions vs. 28±9ms with VNS in the presence noradrenaline (p=0.003). Taken together, this suggests that VNS does not antagonise the electrophysiological action of exogenous noradrenaline in the rabbit ventricle, whilst accentuated antagonism is evident in changes in heart rate. This likely reflects the greater influence of the parasympathetic nervous system on the sinoatrial node. The anti-arrhythmic action of sustained noradrenaline perfusion in LQTS may be attributed to the relative shorter ventricular action potential and reduced action potential triangulation during combined autonomic stimulation.

3.5 Bolus noradrenaline facilitates and exacerbates arrhythmia associated with QT prolongation

Electrical pickup on the ECG recordings during direct sympathetic nerve stimulation prevents accurate assessment of arrhythmia in the isolated innervated rabbit heart. However, noradrenaline injections appear to be a good correlate of sympathetic nerve stimulation. Figure 3 shows the heart rate response to bolus noradrenaline compared with moderate intensity sympathetic nerve stimulation (10Hz, 40V, 2ms pulse duration) in the isolated rabbit heart. The presented data show that the initial heart rate response to noradrenaline is marginally slower than that of direct nerve stimulation (owing to perfusion delay), however, the maximum heart rate and time to peak heart rate response was similar between conditions.

Data from 12 experiments on the influence of sudden adrenergic stress on the incidence of LQTS associated arrhythmia are presented in Figure 4. Injection of a bolus of noradrenaline into the perfusion line acted in a bi-phasic manner- first increasing the risk of arrhythmia in the 10 to 20 second period immediately after injection and then acting to accelerate the underlying heart rate, suppressing arrhythmic events. In stage 1, bolus noradrenaline acted to increase the severity of the observed arrhythmia, with a dependence on the underlying conditions. For instance, noradrenaline commonly triggered EADs

in the absence of pre-existing arrhythmia or caused VT in hearts already exhibiting ectopic activity. As such, VT was more commonly observed with noradrenaline perfusion during high intensities of VNS. In two of twelve hearts, bolus noradrenaline led to periods of sustained VT (>20secs), which in one case degenerated into ventricular fibrillation. The incidence of spontaneous EADs, VT and sustained VT/VF with VNS and VNS plus bolus noradrenaline are presented in Figure 4B-D. 67% of hearts exhibited spontaneous VT with VNS + bolus noradrenaline vs. 33% with VNS alone. Spontaneous EADs were observed in just 1 heart following noradrenaline perfusion in the absence of VNS. The severity of arrhythmic events was increased by combined stimulation, over VNS alone, as evidenced by an increase in the average arrhythmia score (Figure 4E&F).

3.6 Bolus noradrenaline causes a transient increase in action potential duration and triangulation

To investigate the impact of sudden adrenergic stress on ventricular electrophysiology in LQTS we analysed changes in electrophysiological parameters following the injection of a bolus of noradrenaline in baseline conditions (i.e. in the absence of VNS). This was necessitated by the high incidence of arrhythmia during combined autonomic stimulation. These data are presented in Figure 4. Bolus noradrenaline caused a rapid increase in heart rate that recovered to baseline over a 10-minute period (Figure 5A). A transient increase in the duration of the QT interval, ventricular $MAPD_{90}$ and magnitude of action potential triangulation was observed within approximately 10-seconds of noradrenaline injection; followed by a sustained shortening of ventricular repolarisation and reduction of action potential triangulation (Figure 5B-D). This suggests that application of sudden adrenergic stress in LQTS leads to transient pro-arrhythmogenic alterations in ventricular electrophysiology. Indeed, the time course of changes in ventricular repolarisation paralleled the increase in the risk of arrhythmic events immediately following noradrenaline perfusion (during VNS), as well as the suppression of said arrhythmia with sustained adrenergic activation. Dispersion of repolarisation, as assessed from the duration of the TpTe interval, was similar at all time points (Figure 5E). No difference in beat-to-beat variability for QT interval or APD was observed at any time point (data not shown).

4. Discussion

To our knowledge, this is the first study to show the importance of dynamic interplay between the two branches of the autonomic nervous system in LQTS-associated arrhythmogenesis. Our results show that sudden adrenergic stress superimposed on a background of sustained parasympathetic activation is pro-

arrhythmic. However, combined adrenergic and parasympathetic stimulation has a complex relationship with arrhythmogenicity in LQTS. Whilst sustained (steady-state) noradrenaline perfusion suppresses the pro-arrhythmic action of VNS, application of a sudden adrenergic stressor acts to increase the severity of observed events. Noradrenaline rarely triggers spontaneous VT (1/12 hearts) in the absence of VNS, suggesting that concomitant autonomic activation (or "autonomic conflict") is required for the adrenergic facilitation of LQTS-associated arrhythmia. Importantly, this observation parallels the clinical profile for patients with congenital LQTS type 2, who commonly die when awoken suddenly by an alarm clock - suggesting that they experience lethal arrhythmia when a surge in sympathetic / adrenergic activity is superimposed during a time of high parasympathetic tone (i.e. during rest or sleep). [10, 13] By recapitulating LQTS type II, using drugs that block I_{Kr}, we show that the number and severity of arrhythmia observed with VNS alone is exacerbated by application of adrenergic stressor during sustained parasympathetic stimulation. Thus, the facilitation of arrhythmogenesis in LQTS by adrenergic stress is critically dependent upon the timing and sequence of autonomic stimulation. This critical dependence on the sequence of events likely contributes to the stochastic nature of arrhythmia and the difficulties in predicting any individual's risk of sudden death or the timing of that death.

The majority of studies on the influence on adrenergic activation on cardiac electrophysiology have focussed on steady-state responses and there has been only limited investigation into the importance of dynamic changes in autonomic tone in LQTS. In studies in the isolated ventricular wedge preparation, Schimizu *et al.* reported that in LQTS type 2, noradrenaline perfusion leads to a transient increase in transmural dispersion of repolarisation.[16] The authors showed that increased repolarisation heterogeneity correlated with an increase in the susceptibility to spontaneous and electrically induced VT. In our analysis of T-wave duration we found no evidence that adrenergic stress results in an increase in dispersion of repolarisation in the rabbit ventricles, but observed a transient increase in action potential duration and triangulation. The rate of repolarisation is thought to be an important factor in the generation of EADs, as this may lead to the reactivation of the inward calcium current and the reversal of membrane potential - either directly via inward current through the L-type calcium channels or due to stimulated release of calcium from the sarcoplasmic reticulum.[17-19] Thus, the increase in action potential triangulation with sudden adrenergic stress combined with parasympathetic activation may facilitate EADs and explain the transient increase in risk of arrhythmia with concomitant autonomic stimulation. Notably, recurrent ectopic activity is one mechanism thought to underpin *TdP* and is a feasible mechanism for the increased incidence of VT observed during bolus noradrenaline injections.[20] Our observations parallel a report by Liu *et al.*, who demonstrated a transient risk for EADs in isolated

myocytes from rabbits with congenital LQTS type 2 on exposure to isoprenaline - attributed to differential kinetics for activation of the L-type calcium current and slow component of the delayed rectifying potassium current.[21] A similar kinetic mismatch in the targets for protein kinase A dependent phosphorylation was predicted by integrated computational modelling of calcium and beta-adrenergic signalling.[22] Interestingly, in a follow up study, Xie et al. predicted that that sudden adrenergic stress favours the transition from VT to VF, due to dynamic changes in APD restitution, which may have relevance to our observation that bolus noradrenaline is associated with a transient risk of more severe tachyarrhythmia.[23] Faster onset kinetics of the L-type calcium current may explain the transient increase in action potential duration, triangulation and incidence of VT with bolus noradrenaline perfusion in our study. With more sustained adrenergic stimulation, the current carried by I_{Ks} is increased, acting to shorten the action potential and to reduce the risk of further ectopic activity. The same mechanism could explain why sustained adrenergic stimulation acts to suppress arrhythmia. Indeed, loss-of-function mutations in the pore-forming subunits of channels that conduct I_{Ks} are the most common form of congenital LQTS. In this scenario, I_{Ks} is weaker and does not counteract the increase in L-type current during adrenergic-stimulation, promoting EADs. Another factor in the suppression of LQTS-associated arrhythmia by sustained adrenergic stimulation is the resulting acceleration of heart rate, which shortens the action potential, secondary to changes in the concentration of intracellular ions, including, Na and Ca. Bradycardia is a well-established trigger for drug-induced LQTS-related arrhythmia and tachypacing can prevent sudden death both acutely and as a long-term therapy.[24, 25]

That VNS is an independent trigger for arrhythmia in drug-induced LQTS is not surprising and is in keeping with existing clinical and experimental evidence. Bradycardia is a well-established risk factor for TdP and Farkas *et al.* previously demonstrated that the induction of TdP in the phenylephrine-sensitised rabbit is dependent upon activation of the parasympathetic nervous system.[26] Phenylephrine, a selective alpha1-adrenoceptor agonist, increases blood pressure and activates the baroreceptor reflex, leading to a slowing of heart rate via the vagus nerve. In our study, VNS causes prolongation and triangulation of the ventricular action potential, which likely explains the pro-arrhythmogenic action of parasympathetic activation in LQTS. It is notable that one of the best correlates of ectopic activity following VNS was the TpTe interval, a measure of dispersion of repolarisation. This observation could indicate the requirement for a critical gradient of repolarisation in order that ectopic beats can propagate through the ventricular myocardium. Alternatively, the TpTe interval may simply reflect the development of islands of tissue with prolonged APD from where ectopic activity first arises.[27] Furthermore, increased dispersion of repolarisation is a substrate that favours re-entrant

arrhythmia and may predispose to TdP, in keeping with our previous observations.[28]

Beta-adrenoceptor antagonists are the first line therapy for congenital LQTS, with an efficacy that is determined by the molecular basis of QT-prolongation. Beta-adrenoceptor blockade is reported to be most effective in LQTS type 1 and only moderately effective in LQTS type 2 and LQTS type 3.[10] On this basis, we propose that the partial effectiveness of beta-blockade in congenital LQTS type 2 reflects that arrhythmia can be triggered by parasympathetic activation alone, but that the incidence and severity of arrhythmia is increased by sudden adrenergic stress. Beta-blockade likely inhibits the latter case, but clearly this does not entirely abolish the risk of TdP in this patient population. Many useful therapeutic drugs, including antipsychotics and antiarrhythmics, also cause QT prolongation and carry risk of TdP and sudden cardiac death [29]. Where the risk-to-benefit ratio is high, QT-prolonging drugs should be withdrawn in patients exhibiting excessive QT prolongation or symptoms of LQTS (e.g. dizziness, syncope). However, when the risk to benefit ratio is low (i.e. low-risk, high-benefit), our data suggest that patients could benefit from prophylactic beta-adrenoceptor blockade. That is to say, beta-blockade may be an effective strategy to mitigate risk of sudden death in drug-induced LQTS, in addition to congenital forms, and where there is no alternative to use of QT-prolonging drugs. Symptoms of syncope and the risk of sudden death in patients with congenital LQTS who are refractory to beta-blockade can also be mitigated by left sympathetic denervation, a surgical procedure that involves ablation of left stellate ganglion; the major source of sympathetic efferent nerve fibres that innervate the ventricles. Schwartz et al. have previously shown the efficacy of this approach in patients with congenital LQTS who continue with symptoms of syncope or cardiac arrest despite beta-blocker therapy.[30] With type 2 LQTS, where bradycardia is an independent trigger for arrhythmia, further benefits could be provided by the combined use of beta-blockers/denervation and chronic atrial pacing.[25]

We have previously suggested that autonomic conflict, the simultaneous activation of the sympathetic and parasympathetic inputs to the heart, might be an arrhythmogenic trigger.[3] The basis for this is observations of substantive cardiac dysrhythmia and arrhythmia in young healthy adults within the first minute of submersion in cold-water; a potent stimulator of the sympathetically driven cold-water shock response and parasympathetcally mediated mammalian diving reflex. Importantly, similar events are not commonly observed in the same participants during head out cold-water immersion, or with facial immersion alone, suggesting that autonomic co-activation plays a critical role. We recently reported that combined autonomic stimulation (i.e. sustained noradrenaline + VNS) is insufficient to cause significant arrhythmia in healthy isolated rabbit hearts.[31] However, we show presently that

concomitant stimulation can trigger severe arrhythmia in the presence of a predisposing electrophysiological abnormality, and in a manner, that is critically dependent on the timing of the adrenergic stimulus. Autonomic conflict may also have broader importance in other forms of LQTS, for example, it may explain why swimming is a gene-specific trigger for TdP and sudden death in congenital LQTS type 1.[9] Moreover, dynamic events, leading to sudden alterations in autonomic tone, are likely to be an important contributing factor in many arrhythmogenic syndromes and pre-disposing conditions. For example, swimming may be a trigger for arrhythmia in patients with genetic mutations associated with catecholinergic polymorphic ventricular tachycardia.[11] Greater understanding of how the dynamic changes in autonomic tone influence electrophysiology and arrhythmogensis in disease may be an important factor in the development of more effective therapeutic strategies for the management of sudden cardiac death.

Animal models of TdP are essential for studies of the arrhythmogenic processes in LQTS, and also have utility for safety screening of QT prolonging drugs, however, there are several limitations with existing experimental approaches. These include: the necessary use of anaesthetic agents in *in vivo* models,[26] a failure to predict the pro-arrhythmic potential of recognised torsadegenic drugs,[26] use of non-physiological conditions (e.g. perfusion of acetylcholine to mimic VNS), a reliance on expensive protocols (e.g. chronic atrioventricular block in the dog),[32] and the low incidence of TdP in the absence of electrical induction protocols.[16] In this study, we describe a novel protocol that recapitulates LQTS-associated arrhythmogenesis, including spontaneous EADs and VT. With concomitant autonomic stimulation (VNS + bolus noradrenaline) the maximum incidence of EADs and VT in our study was 92% and 67%, respectively. As such, the innervated rabbit heart may be a useful model for the study of LQTS-associated arrhythmia. This approach has several potential advantages over existing models, including; the preservation of natural cardiac activation and repolarisation patterns, the ability to perform repeat measurements in the same heart, no requirement for anaesthetic agents, that heart rate can be altered physiologically by direct nerve stimulation and great flexibility / adaptability (e.g. rapid washout of drugs, altered ionic composition). Moreover, the model is relatively low-cost and requires only minimal training to implement. This facility of the *ex vivo* innervated heart in drug safety screening will require extensive validation and is a focus for future work.

4.1 Limitations

There are several limitations that deserve discussion. Firstly, in preliminary experiments we found that direct sympathetic nerve stimulation caused significant electrical pickup on ECG recordings, making it difficult to accurately determine the onset and type of arrhythmia. For this reason, we used exogenous noradrenaline, which may not be directly akin to nerve stimulation,

where the “fright” response is likely mediated by local neurotransmitter release. Secondly, both ketamine and medetomidine can influence autonomic control. However, as experiments were performed in an *in vitro* preparation, with significant time for drug washout, we believe this is unlikely to influence our results, and nerve responses were robust and reproducible throughout the experimental protocols. Thirdly, the incidence of VT at baseline between protocols 1 and 2 was modestly different (2 vs. 4 episodes), which could simply reflect that more extended periods of VNS (as in protocol 1) are associated with a higher incidence of spontaneous arrhythmia. However, given that difference between the groups is not statistically significant, this observation may simply be down to chance. Fourthly, on the slowing of heart rate with VNS, it was common for map recordings to exhibit unstable diastolic potentials (when previously stable at baseline), which was likely attributed to slight movement of the recording electrode secondary to altered contractile motion of the heart. This can be seen in the example trace shown in Figure 1A. To avoid mechanical triggering of arrhythmia, we opted not to re-orientate the electrode mid protocol. However, despite the observed instability, it was still possible to accurately detect the onset of EADs, particularly as our analysis relied on concomitant observations in MAP recordings and in the ECG. Finally, clofilium is an K channel blocker with a similar IC_{50} to E4031 and dofetilide, and has proven efficacy for the induction of TdP in rabbits.(ref) The purpose of the present study was to develop a model that replicates congenital LQTS and not necessarily to compare directly to clinically used (or historical) pharmaceutical agents (e.g. d-sotalol, dofetilide). Whilst alternative class III agents are more commonly employed in experimental studies, many K channel blockers exhibit similar potency and we do not believe that our use of clofilium detracts from the conclusions of the present study.[33] Moreover, we have previously shown that VNS can trigger TdP in rabbit hearts treated with E4031, a result replicated with clofilium.[28]

5. Conclusions

Autonomic conflict, the simultaneous activation of parasympathetic and sympathetic autonomic inputs to the heart, can facilitate arrhythmogenesis in LQTS. *Dynamic* alterations in autonomic tone may be an important contributory factor in many arrhythmogenic syndromes or in conditions that predispose to ventricular arrhythmia.

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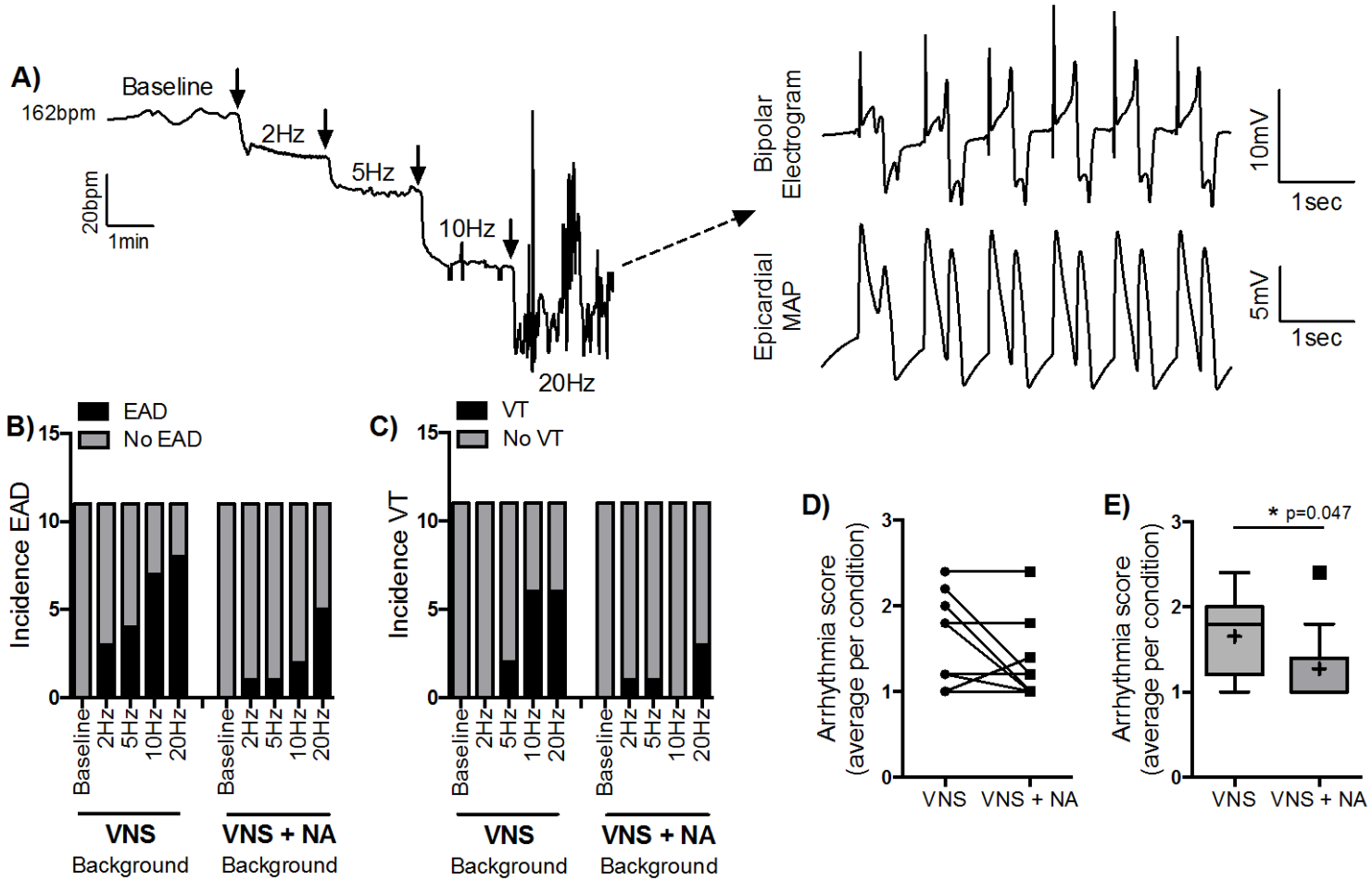

Figure 1. Sustained noradrenaline perfusion suppresses LQTS associated arrhythmia. A) Representative traces demonstrating the slowing of heart rate with increasing intensities of vagus nerve stimulation (VNS) in a clofilium-treated (100nmol/L) rabbit heart. In this example, early afterdepolarisations (EADs) are triggered at 20Hz VNS. B&C) Arrhythmia counts for 11-experiments demonstrating a reduction in the incidence of EADs and ventricular tachycardia (VT) with sustained noradrenaline (NA, 100nmol/L) perfusion. D&E) Individual and average (Tukey's box plot) arrhythmia scores during VNS and with VNS+NA (sustained). Different from VNS alone; *p<0.05. Comparisons by Wilcoxon's matched-groups signed rank-sum test (n=11).

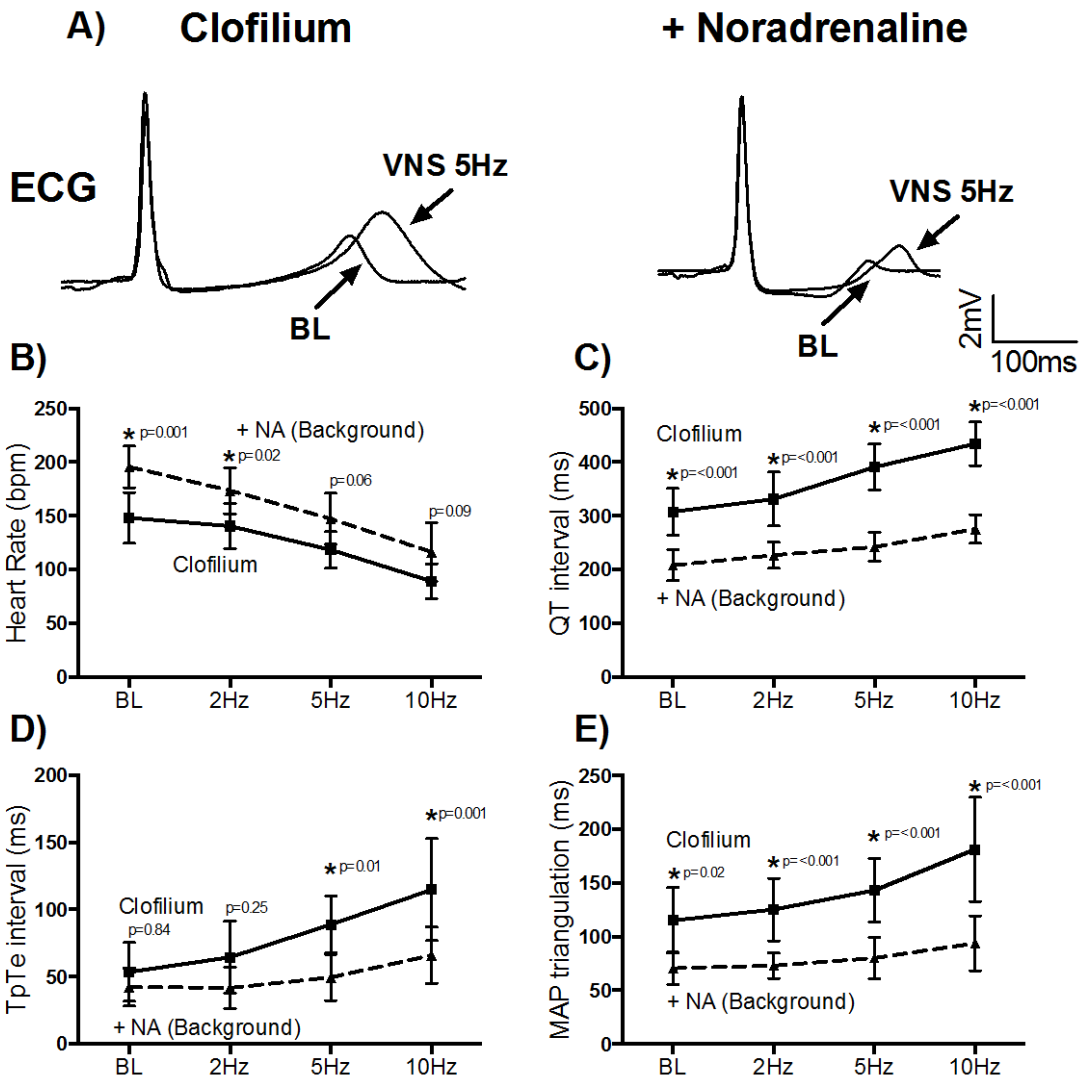

Figure 2. Electrophysiological changes during VNS with and without sustained noradrenaline. A) Representative traces demonstrating changes in electrocardiogram (ECG) parameters during VNS in a clofilium-treated (100nmol/L) rabbit heart, with and without sustained noradrenaline (NA, 100nmol/L) perfusion. B-E) Average data on the change in heart rate, QT interval, T-wave peak to end interval (TpTe) and monophasic action potential (MAP) triangulation. Data represent means (SD). Different from clofilium (control); *p<0.05. Two-way repeated measures ANOVA, with Sidak's post-hoc tests (n=7).

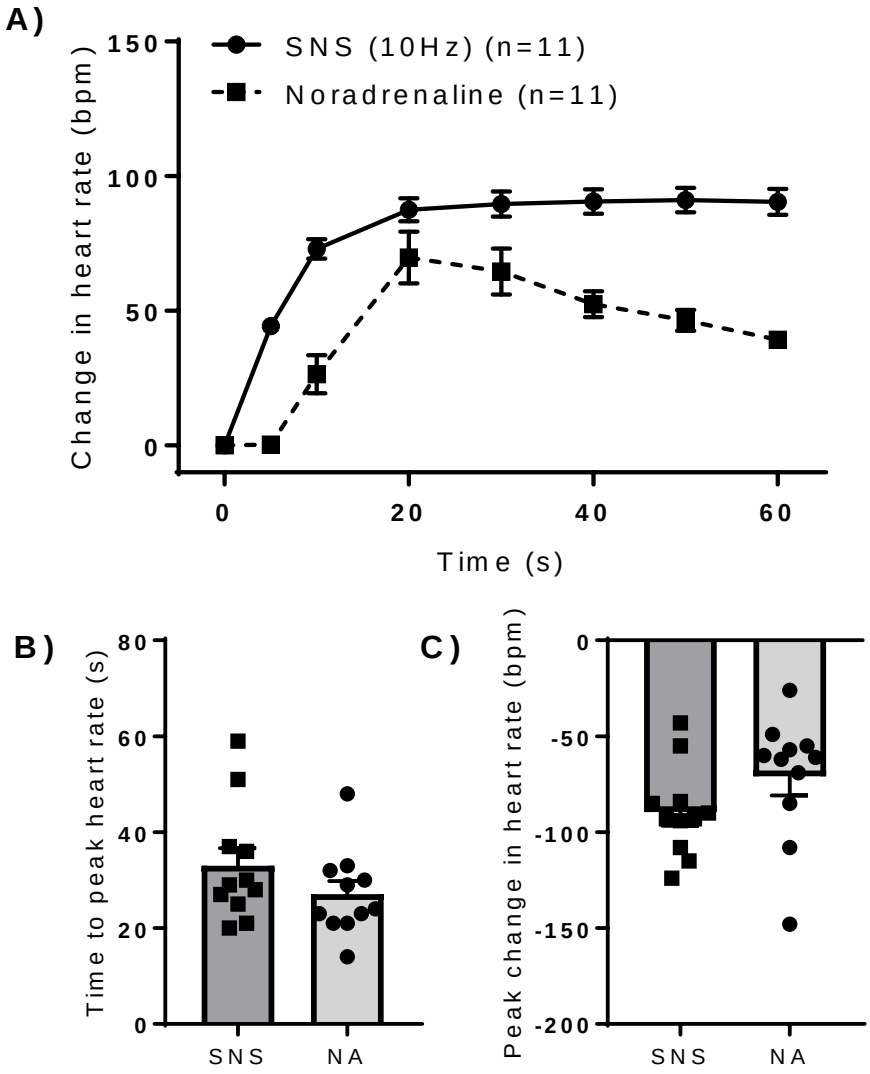

Figure 3. Comparison of heart rate response to sympathetic nerve stimulation and bolus noradrenaline injection. A) Temporal profile of the change in heart rate with bilateral electrical stimulation of the cardiac sympathetic efferent nerves (10Hz, 2ms pulse width, 40V) and with injection of 0.1ml of 1mM noradrenaline stock solution into the perfusion line. B&C) Mean data on the time to peak heart rate responses and the change in heart rate with sympathetic nerve stimulation (SNS) and noradrenaline bolus injection.

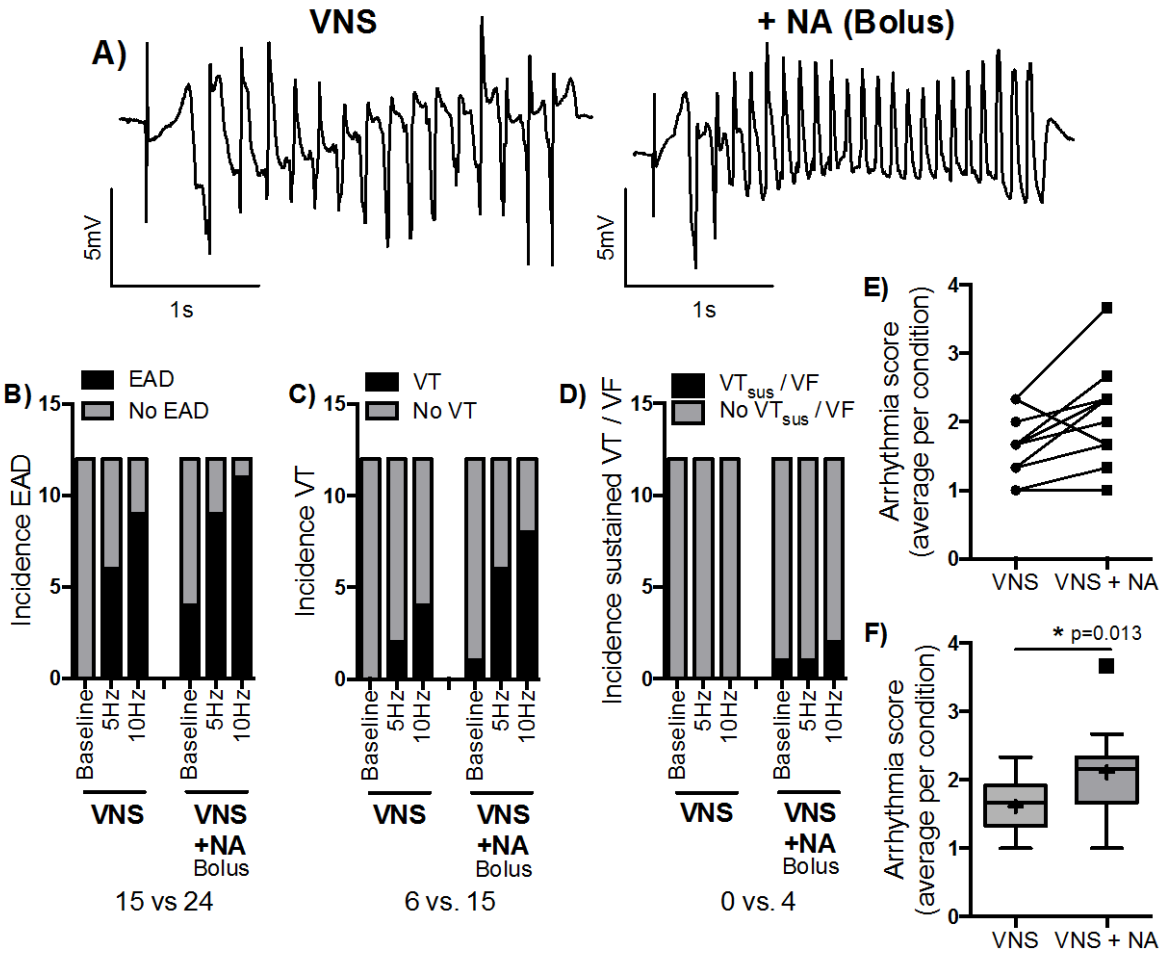

Figure 4. Bolus noradrenaline facilitates LQTS associated arrhythmia. A) Representative episodes of spontaneous ventricular tachycardia (VT) in a clofilium-treated (100nmol/L) rabbit heart. The left trace was observed during VNS and the right following bolus noradrenaline (NA) perfusion (0.1ml of 1mM stock), in separate experiments. B-D) Arrhythmia counts for 12-experiments, demonstrating an increase in the incidence of early aftedepolarizations (EADs), VT and sustained VT (VT_{sus})/ventricular fibrillation (VF) with VNS + NA (bolus). E&F) Individual and average arrhythmia scores during VNS and with VNS+NA (bolus). Different from VNS alone; *p<0.05. Comparisons by Wilcoxon's matched-groups signed rank-sum test (n=12).

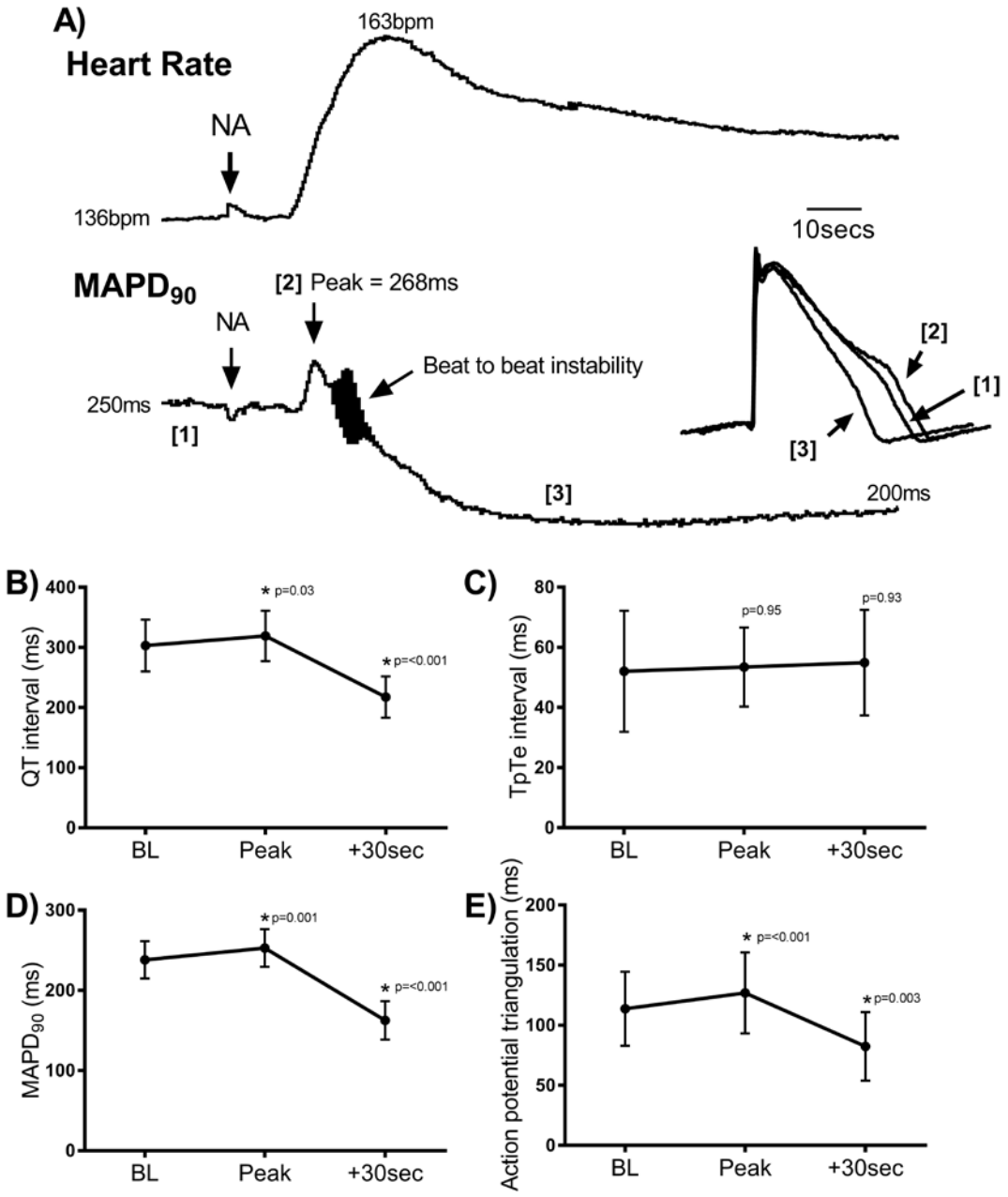

Figure 5. Electrophysiological effects of bolus noradrenaline in LQTS. A) Experimental trace demonstrating the change in heart rate and monophasic action potential duration ($MAPD_{90}$) following bolus noradrenaline (NA) perfusion (0.1ml of 1mM stock) in a clofilium treated (100nmol/L) rabbit heart. A transient increase in $MAPD_{90}$ is seen immediately following NA perfusion. Inset are representative action potential recordings from the timepoints noted by []. B-E) Average data of QT interval, T-wave peak to end interval (TpTe), $MAPD_{90}$ and action potential triangulation at baseline, at peak response and at 30-seconds post-peak response. Data represent means(SD). Different from baseline; *p<0.05. Comparisons by one-way ANOVA, with Sidak's post-hoc tests (n=9).

Table 1. Electrophysiological predictors of early afterdepolarisations.

Parameters	Experiments without EADs	Experiments with EADs	P value
RR interval (ms)	465 (98)	560 (105)	0.139
QT Interval (ms)	335 (38)	382 (50)	0.098
TpTe Interval (ms)	59 (10)	99 (21)	*0.001
APD_{90} (ms)	261 (26)	285 (42)	0.176
APD BBV (ms)	2.8 (3.9)	12.3 (24.0)	0.364
Action potential triangulation (ms)	125 (17)	164 (39)	*0.045

Comparison of electrocardiogram and monophasic action potential parameters in hearts with and without early afterdepolarisations (EADs) during vagus nerve stimulation (VNS) at 5Hz. 10-beat averages immediately before the first EAD or the last 10-beats before the cessation of VNS. BBV=beat-to-beat variability. Data represent means (SD). Comparisons by paired Student's t-tests. *$p<0.05$, (n=6 per group).